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Time-Resolved Determination of the CcpA Regulon of *Lactococcus lactis* subsp. *cremoris* MG1363▽

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Carbon catabolite control protein A (CcpA) is the main regulator involved in carbon catabolite repression in gram-positive bacteria. Time series gene expression analyses of *Lactococcus lactis* MG1363 and *L. lactis* MG1363Δ*ccpA* using DNA microarrays were used to define the CcpA regulon of *L. lactis*. Based on a comparison of the transcriptome data with putative CcpA binding motifs (*cre* sites) in promoter sequences in the genome of *L. lactis*, 82 direct targets of CcpA were predicted. The main differences in time-dependent expression of CcpA-regulated genes were differences between the exponential and transition growth phases. Large effects were observed for carbon and nitrogen metabolic genes in the exponential growth phase. Effects on nucleotide metabolism genes were observed primarily in the transition phase. Analysis of the positions of putative *cre* sites revealed that there is a link between either repression or activation and the location of the *cre* site within the promoter region. Activation was observed when putative *cre* sites were located upstream of the hexameric −35 sequence at an average position of −56.5 or further upstream with decrements of 10.5 bp. Repression was observed when the *cre* site was located in or downstream of putative −35 and −10 sequences. The highest level of repression was observed when the *cre* site was present at a defined side of the DNA helix relative to the canonical −10 sequence. Gel retardation experiments, Northern blotting, and enzyme assays showed that CcpA represses its own expression and activates the expression of the divergently oriented prolidase-encoding *pepQ* gene, which constitutes a link between regulation of carbon metabolism and regulation of nitrogen metabolism.

Carbon catabolite repression (CCR), a well-known regulatory mechanism by which bacteria regulate the metabolism of carbon and other energy sources, has been the subject of intense study for the last three decades. In gram-positive bacteria, CCR is mediated by carbon catabolite control protein A (CcpA) (57). The unrelated transcription factor cyclic AMP receptor protein (CRP) is responsible for CCR in gram-negative bacteria (40). In addition to CCR, CcpA has been found to be required for positive regulation (carbon catabolite activation) of the expression of genes encoding enzymes involved in glycolysis in *Bacillus subtilis* (4) and *Lactococcus lactis* (32).

Positive and negative regulation of the transcription of CcpA-regulated genes involves the binding of CcpA to *cis*-acting catabolite responsive elements (*cre* sites) (47). The binding of CcpA to a *cre* site is strongly stimulated by HPr when the latter compound is phosphorylated at Ser46 (HPr-Ser46p) (45). CcpA binds to DNA as a heterotetramer with one HPr-Ser46p protein per CcpA subunit (45). A second HPr-like protein in *B. subtilis*, Crh, has been shown to be functional in CCR and could partially take over the function of HPr (46). It has been suggested that Crh might be specifically involved in repression of metabolic genes by nonsugars (56).

Different mechanisms have been suggested for the repression of target genes in *B. subtilis* by CcpA; these mechanisms include inhibition of transcription initiation (13, 24) and "roadblocking" of the transcription machinery (6, 19). Recently, it has been reported that *B. subtilis* CcpA interacts with RNA polymerase (RNAP), causing inhibition of transcription initiation (25). For this interaction to occur, the *cre* site must be present at a specific face of the DNA helix. Previous research has shown that for transcriptional activation of *ackA* by CcpA in *B. subtilis*, a *cre* site must be present upstream of the −35 sequence in the *ackA* promoter, specifically at position −56.5 (49). Transcriptional activation of the *ackA* gene by CcpA was abolished when a 5-bp fragment was inserted between *cre* and the *ackA* promoter, while insertion of a 10-bp fragment did not have a negative effect on transcription (49). This phenomenon has also been demonstrated for class I activators in *Escherichia coli*, where the activator binding site must be on the same face of the DNA helix as the RNAP binding site to achieve transcriptional activation. It has been shown that for transcriptional activation of the *ppsA* gene by the CcpA homolog Cra in *E. coli*, the Cra binding site must be centered at position −45.5 (38).

Less is known about carbon catabolite control by CcpA in *L. lactis*. The known targets of *L. lactis* CCR are the *gal* operon for galactose utilization (32), the *fru* operon for fructose utilization (3), and the *noxE* gene (NADH oxidase) (12). Furthermore, it has been proposed by Guedon et al. (16) that lactococcal *pepP* (aminopeptidase P) is regulated by CcpA. A known target of *L. lactis* carbon catabolite activation is the *las* operon (32), and it has been suggested that CcpA also activates

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype or genotype	Reference or source
L. lactis strains		
MG1363	*L. lactis* subsp. *cremoris*, plasmid-free derivative of NCDO712	11
MG1363Δ*ccpA*	MG1363, chromosomal deletion of *ccpA*	This study
E. coli strains		
EC1000	Km^r, *glgB*, derivative of MC1000 containing *repA* gene of pWV01	28
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q} *ZΔM15* Tn*10* (Tet^r)]	Stratagene
Plasmids		
pORI280	Em^r, β-Gal$^+$, *ori*$^+$ of pWV01; integration vector that replicates in strains containing *repA*	28
pVE6007	Cm^r, pWV01 derivative encoding a temperature-sensitive RepA protein	33
pORI280::Δ*ccpA*	pORI280 derivative carrying *ccpA* up- and downstream regions	This study
pILORI4	Em^r, pIL252 carrying the multiple cloning site and promoterless *lacZ* of pORI13	27
pILORIAZ	pILORI4 carrying 280-bp *ccpA* promoter fragment	This study
pILORIQZ	pILORI4 carrying 280-bp *pepQ* promoter fragment	This study
pQE30-ccpA	Amp^r, pQE30 carrying *ccpA* from *L. lactis* IL1403	26

the expression of the *fhuR* (heme uptake regulator) regulatory protein under respiration-permissive conditions (12).

Since CcpA has been shown to be able to act both as a repressor and as an activator, analysis of the exact locations of the *cre* sites relative to the RNAP binding sites and a comparison of the in silico data obtained with in vivo experiments involving, for example, transcriptome analysis could provide new insights into the activation and repression mechanisms of CcpA.

CcpA-mediated CCR in *B. subtilis* has been extensively studied using various techniques, including DNA microarray analysis and proteomics techniques. A genome-wide transcriptome comparison of a wild-type strain and a *ccpA* knockout mutant of a lactic acid bacterium has not been reported yet. As lactic acid bacteria (LAB) live in natural environments (e.g., milk, silage, and gastrointestinal tracts) that are substantially different from the environment in which *B. subtilis* normally resides (soil), we anticipate that there are differences in the types of genes regulated by the CcpA proteins of these organisms. For *Streptococcus thermophilus*, which commonly resides in the same niche as *L. lactis*, the importance of the CcpA protein for adaptation to growth in milk and for the use of lactose as the primary carbon source has already been shown (52).

Completion of the genome sequence of *L. lactis* MG1363 (U. Wegmann, M. O'Connell-Motherway, A. L. Zomer, et al., unpublished results) allowed us to perform full genome transcriptome analyses of *L. lactis* MG1363 using *L. lactis* MG1363 DNA microarrays. This nucleotide sequence also made it possible to search for possible *cre* sites in the coding and noncoding regions of the *L. lactis* MG1363 genome.

In transcriptome studies of carbon catabolite control by the CcpA protein in other gram-positive bacteria, workers have focused on regulation of gene expression in the exponential growth phase (4, 31, 37, 58). We expected to be able to extract more information by studying the transcriptome in all growth phases, and we focused on CcpA-mediated regulation of *L. lactis* MG1363 gene expression not only in the exponential phase but also at the onset of the exponential phase, at the transition between the exponential growth phase and the stationary phase, and in the stationary phase.

We found that CcpA is a key regulator in *L. lactis* and that both the "interaction" mechanism and the "roadblocking" mechanism of gene regulation may play significant roles in CcpA-mediated carbon catabolite repression in *L. lactis*. Furthermore, we identified a link between carbon metabolism regulation and nitrogen metabolism regulation via activation of *pepQ* by CcpA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Relevant information concerning the plasmids and strains used in this study is shown in Table 1. *L. lactis* was grown at 30°C or 38°C in GM17 medium (Difco Laboratories, Detroit, MI) as standing cultures or on agar plates containing 1.5% (wt/vol) agar. *L. lactis* was also grown in chemically defined medium (CDM) (21) supplemented with Casitone (Difco Laboratories) at a concentration of 0.2% or 2% (wt/vol). All media were supplemented with 0.5% (wt/vol) glucose, while 4 μg/ml chloramphenicol (Sigma Chemical Co., St. Louis, MO), 5 μg/ml erythromycin (Roche Molecular Biochemicals, Mannheim, Germany), or 0.008% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma Chemical Co.) was added when appropriate. *Escherichia coli* was grown in TY medium (Difco Laboratories) at 37°C with vigorous agitation or on TY medium solidified with 1.5% agar; 100 μg/ml erythromycin or 100 μg/ml ampicillin (Roche Molecular Biochemicals) and 0.002% X-Gal were added when necessary.

DNA techniques and transformation. Molecular cloning techniques were performed essentially as described by Sambrook et al. (43). Restriction enzymes, T4 DNA ligase, Expand DNA polymerase, *Taq* DNA polymerase, and PWO DNA polymerase (Roche Molecular Biochemicals) were used according to the instructions of the supplier. Synthetic oligonucleotides were synthesized at Isogen Life Science (Ijsselstein, The Netherlands). PCR products were purified using a High Pure PCR product purification kit (Roche Molecular Biochemicals). Plasmid DNA was introduced into *E. coli* and *L. lactis* by electrotransformation as described by Zabarovsky et al. (59) and Leenhouts et al. (29), respectively. Analytical grade chemicals were obtained from Merck (Darmstadt, Germany) or BDH (Poole, United Kingdom).

Construction of a *ccpA* mutant of *L. lactis*. The *ccpA* deletion mutation was introduced into *L. lactis* subsp. *cremoris* MG1363 using plasmids pVE6007 (33) and pORI280 (28) in a double-crossover procedure, as described by Leenhouts et al. (28). *E. coli* EC1000 (RepA$^+$) was used as the intermediate host during vector construction. The *ccpA* upstream region was amplified with primers RLTRXB1 and RLCCPA1 (see Table P1 posted at http://molgen.biol.rug.nl/publication/ccpA_data) using Expand polymerase and was cloned as an XbaI/BamHI fragment in the corresponding sites in pORI280. The *ccpA* downstream region was amplified using primers HE74 and HE51 (see Table P1 posted at http://molgen.biol.rug.nl/publication/ccpA_data) and was cloned as a BamHI/EcoRI fragment in the vector carrying the upstream fragment, resulting in plasmid pORI280::Δ*ccpA*. The deletion plasmid was introduced into *L. lactis* MG1363(pVE6007). The deletion strain was obtained in two steps as described previously (28). First, integration and deletion of helper plasmid pVE6007 were

performed at the nonpermissive temperature (38°C) and were confirmed by Southern blotting, colony PCR, and determination of the sensitivity of the strains to chloramphenicol; second, excision and deletion of the integration vector were performed by growth of the integrant in the absence of antibiotic for at least 100 generations at 30°C. The genetic structure of the resulting *ccpA* deletion strain, *L. lactis* MG1363Δ*ccpA*, was confirmed by colony PCR, Southern blotting, and determination of the sensitivity of the strain to erythromycin.

Cloning of *ccpA* and *pepQ* promoter fragments. Oligonucleotides used to amplify the region between the divergent *ccpA* and *pepQ* genes are shown in Table P1 posted at http://molgen.biol.rug.nl/publication/ccpA_data. Primers ccpA-pepQ-fw and ccpA-pepQ-rev were used to amplify chromosomal DNA of *L. lactis* MG1363, which yielded the promoter regions of *ccpA* and *pepQ*. The PCR product was cut with XbaI and was inserted into the corresponding site upstream of the promoterless *lacZ* gene in pILORI4 (27), and the ligation mixture was used to transform *L. lactis* MG1363. Plasmids containing the *ccpA-pepQ* intergenic region in both orientations were obtained and were introduced into *L. lactis* MG1363Δ*ccpA*. The plasmid with the *ccpA* promoter and the plasmid carrying the *pepQ* promoter upstream of *lacZ* were designated pILORIAZ and pILORIQZ, respectively.

Northern blotting and primer extension. RNA was isolated from exponentially growing *L. lactis* cells at an optical density at 600 nm of 0.5 as previously described (50). Northern blot analysis was performed as described previously (42) with PCR products generated using primers pepq_avrnco and pepq_spe and primers RL16srna1 and RL16srna2 (see Table P1 posted at http://molgen.biol.rug.nl/publication/ccpA_data). A synthetic oligonucleotide (pepQTS2 [see Table P1 posted at http://molgen.biol.rug.nl/publication/ccpA_data]) complementary to *pepQ* mRNA was used for primer extension analysis. Two picomoles of primer was added to 5 μg of total RNA in a reaction mixture containing 0.5 mM dCTP, 0.5 mM dGTP, 0.5 mM dTTP, 0.05 mM dATP, and α-^{35}S-labeled dATP. cDNA was synthesized using SuperScript transcriptase (Roche Molecular Biochemicals). Following 50 min of incubation at 42°C, the reaction was stopped by heating the mixture at 70°C for 15 min. The primer extension product was compared on a sequencing gel with the products of a sequencing reaction in which the same primer was used.

Expression and purification of H6-CcpA. His-tagged CcpA from *L. lactis* IL-1403 (H6-CcpA) was purified from *E. coli* essentially as described previously (26), with the following modifications. An overnight culture of *E. coli* strain XL1-Blue with a plasmid expressing H6-CcpA was diluted 1:50 into fresh TY medium supplemented with 100 μg of ampicillin/ml and grown at 37°C with vigorous shaking. At an A_{600} of 0.6, expression of the recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG) (Roche Molecular Biochemicals). The culture was incubated for an additional 4 h, after which the cells from 600 ml of the culture were collected by centrifugation (10 min, 8,000 rpm, 4°C) with an Avanti J-20 XP centrifuge (Beckmann Coulter, Mijdrecht, The Netherlands). The pellet was washed with 50 ml of buffer A (50 mM $NaHPO_4$, 300 mM NaCl, 10 mM imidazole, 3.5% glycerol, 1 mM β-mercaptoethanol; pH 8.0) and stored at −80°C until it was used. The pellet was resuspended in 10 ml of buffer A, and the cells were disrupted by sonication. Cellular debris was removed by centrifugation (30 min, 20,000 g, 4°C), and the supernatant fraction was purified as described previously (26). The purified protein sample (1 ml) was dialyzed three times in 1 liter of a buffer containing 10% glycerol, 100 mM NaCl, and 20 mM Tris-HCl (pH 8) at 0°C to remove excess salts. The purified protein was examined to determine its protein content and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and to determine its DNA content by ethidium bromide staining on agarose gels. The protein was quantified using an RC/DC protein determination kit according to the instructions of the supplier (Bio-Rad Laboratories, Richmond, CA).

EMSA. DNA fragments containing the *ccpA* and *pepQ* promoters were prepared by PCR using primers ccpA-pepQ-fw and ccpA-pepQ-rev (see Table P1 posted at http://molgen.biol.rug.nl/publication/ccpA_data). Electrophoretic mobility shift assays (EMSAs) were performed essentially as described previously (18). The PCR products were end labeled with T4 polynucleotide kinase (Roche Molecular Biochemicals) and [γ-^{32}P]ATP. The protein and probe were mixed on ice and subsequently incubated for 20 min at 30°C. Samples were loaded onto 6% nondenaturing polyacrylamide gels prepared in TAE buffer (40 mM Tris acetate [pH 8.0], 2 mM EDTA) and electrophoresed in a 0.5× to 2.0× gradient of TAE buffer at 100 V for 60 min in a Mini Protean electrophoresis unit (Bio-Rad Laboratories). The gels were dried with a vacuum dryer (model 583; Bio-Rad Laboratories), and signals were recorded using phosphoscreens and a Cyclone PhosphorImager (Packard Instruments, Meridien, CT).

DNA microarray experimental procedures. DNA microarrays containing amplicons of 2,457 annotated genes in the genome of *L. lactis* subsp. *cremoris* MG1363 were designed and made as described previously (54). Slide spotting, slide treatment after spotting, and slide quality control were performed as described previously (53). Samples used for RNA isolation were obtained by rapid sampling of early- and mid-exponential-phase, transition-phase, and stationary-phase cultures of *L. lactis*. Cell disruption, RNA isolation, RNA quality control, cDNA (target) synthesis, indirect labeling, hybridization, and scanning were performed as described by van Hijum et al. (53).

Statistical procedures. DNA microarray data were processed as previously described (9, 53, 55), with the following modifications. The minimum and maximum numbers of measurements for each gene were five and eight (i.e., one experimental condition for which four independent RNA isolations were performed), respectively.

Differential expression tests were performed with the Cyber-T implementation of a variant of the *t* test (30). False discovery rates were calculated as described previously (53). A gene was considered differentially expressed when the *P* value was <0.001, the false discovery rate was <0.05, and ratio was >2 or <0.55. The DNA microarray data are available at http://molgen.biol.rug.nl/publication/ccpa_data.

Promoter predictions. Locations of SigmaA binding sites in the genome sequence of *L. lactis* MG1363 were predicted using hidden Markov models of the SigmaA binding site, allowing 15 to 19 bp of space between the canonical −35 and −10 promoter elements. The models were based on known Sigma A binding sites (22, 51) (see the data at http://molgen.biol.rug.nl/publication/ccpa_data). The presence of *cre* sites was examined using a weight matrix based on known *cre* sites (2) with the MotifLocator tool (1). Promoter searches were performed for sequences up to 400 bp upstream of the translational start site of each gene and for the sequences of all open reading frames present in the *L. lactis* MG1363 genome. Locations of *cre* sites were calculated on the basis of the position of the hexameric −10 region. When possible, the predicted −35 and −10 regions were compared with previously published data for transcriptional start site (TSS) determinations and were adjusted accordingly.

Enzyme assays. β-Galactosidase activity assays were performed using cell suspensions from exponentially growing *L. lactis* cultures. Cells were permeabilized with chloroform as described previously (20). For prolidase activity assays cell extracts were prepared as described previously (44), and prolidase enzyme activity was measured essentially as described previously (39).

RESULTS

Global analysis of the *L. lactis* MG1363Δ*ccpA* transcriptome using functional categories. To identify the genes whose expression in rich medium with glucose (GM17) changed when the *ccpA* gene was deleted, the transcriptional profile of *L. lactis* MG1363 was compared to that of *L. lactis* MG1363Δ*ccpA* in four distinct phases of growth, the early exponential growth phase, the mid-exponential growth phase, the transition phase between the exponential and stationary growth phases, and the stationary phase of growth. Although the final turbidities of *L. lactis* MG1363 and *L. lactis* MG1363Δ*ccpA* cultures were identical and the optical densities at 600 nm were as high as 2.2, deletion of the gene encoding the CcpA protein had a significant effect on the rate of growth of *L. lactis* in GM17. Therefore, samples used for RNA isolation were taken from the cultures at comparable growth phases (Fig. 1). The results of the analysis of the transcriptomes of the two strains at the four times during growth are shown in Table 2, which shows that repression by CcpA occurred more frequently than activation occurred. The strongest effect of deletion of *ccpA* was observed in the transition phase since most genes were affected by the *ccpA* mutation in this growth phase.

So that we could interpret the results more globally, the genes were grouped on the basis of the putative functions of the encoded proteins (48). Most of the affected genes were in the COG functional category "carbohydrate transport and metabolism." A large number of genes in the COG functional category "amino acid transport and metabolism" were also affected by the absence of CcpA, suggesting that the protein

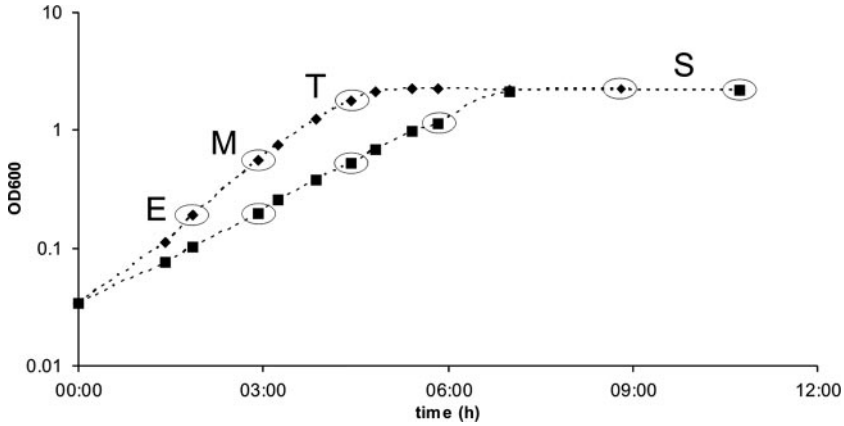

FIG. 1. Growth of *L. lactis* and its isogenic *ccpA* mutant in GM17: growth curves for *L. lactis* MG1363 (◆) and *L. lactis* MG1363Δ*ccpA* (■). Times at which samples were collected for RNA isolation in the early exponential phase (E), the mid-exponential phase (M), the transition phase between the exponential and stationary phases (T), and the stationary phase (S) are circled. OD600, optical density at 600 nm.

also has an effect on the regulation of nitrogen metabolism and proteolysis (Table 2).

It is clear that CcpA activity occurred mainly in and around the exponential growth phase (early exponential phase, mid-exponential phase, and transition phase), while the role of CcpA in the stationary phase of growth was reduced. This observation is in accordance with the absence of *ccpA* mRNA in *L. lactis* MG1363 growing in the stationary phase, which is clear from the absolute levels of expression of *ccpA* on the DNA microarrays (see Table S1 posted at http://molgen.biol.rug.nl/publication/ccpA_data).

Growth phase-dependent regulation by CcpA. Although there was a major overlap between the transcriptome in the exponential growth phase and the transcriptomes in other growth phases, a large number of genes were regulated exclusively in one growth phase (Fig. 2). Deletion of *ccpA* changed the expression of 49 genes exclusively in the early exponential phase, 24 genes exclusively in the mid-exponential growth

TABLE 2. Numbers of genes significantly affected by deletion of *ccpA* in *L. lactis* in different COG categories[a]

Functional category	No. of genes affected							
	Early exponential phase		Mid-exponential phase		Transition phase		Stationary phase	
	Up-regulation	Down-regulation	Up-regulation	Down-regulation	Up-regulation	Down-regulation	Up-regulation	Down-regulation
J. Translation, ribosomal structure, and biogenesis	0	2	1	2	6	2	0	5
K. Transcription	3	2	3	2	7	3	1	2
L. DNA replication, recombination, and repair	1	0	0	0	4	3	0	1
D. Cell division and chromosome partitioning	0	0	0	0	0	0	0	0
V. Defense mechanisms	2	0	2	0	0	3	0	0
O. Posttranslational modification and protein turnover	0	0	1	1	1	2	2	4
M. Cell envelope biogenesis, outer membrane	2	0	1	2	1	0	1	0
P. Inorganic ion transport and metabolism	1	5	2	0	5	2	0	3
U. Intracellular trafficking and secretion	0	0	0	1	0	2	0	1
N. Cell motility	0	0	0	0	0	0	0	0
T. Signal transduction mechanisms	3	0	3	0	2	2	0	2
F. Nucleotide transport and metabolism	0	9	0	1	15	0	0	1
G. Carbohydrate transport and metabolism	28	10	22	1	12	9	2	10
E. Amino acid transport and metabolism	11	4	16	2	9	11	0	8
H. Coenzyme metabolism	2	2	3	1	3	4	0	2
I. Lipid metabolism	2	0	2	0	2	3	0	0
C. Energy production and conversion	8	0	3	0	4	3	3	7
Q. Secondary metabolite transport and metabolism	1	0	1	0	2	0	0	0
W. Extracellular structures	0	0	1	0	1	0	0	0
R. General function prediction only	7	2	7	2	12	1	1	3
S. Function unknown	3	2	3	0	3	4	1	4
X. No prediction	16	0	11	1	5	9	1	8
Total[b]	85	33	70	16	88	60	12	58

[a] For upregulation there was at least a 2-fold increase and for downregulation there was at least a 1.8-fold decrease in the level of expression, and the data met the statistical criterion of a Bayesian *P* value of less than 0.001 according to the Cyber-*t* test.

[b] Some genes are assigned to multiple categories.

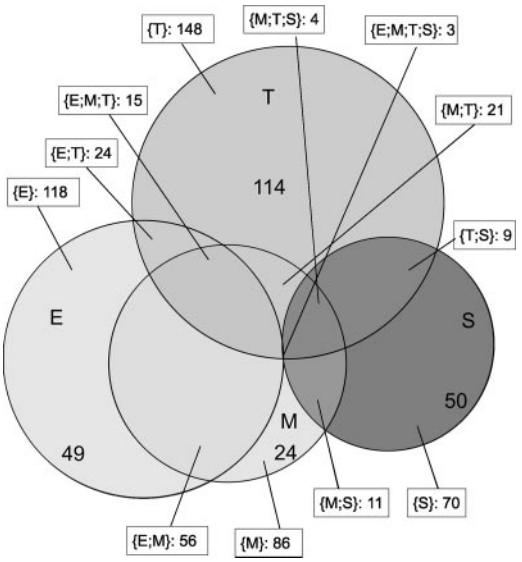

FIG. 2. Venn diagram of genes significantly differentially expressed in *L. lactis* MG1363Δ*ccpA* and its parent during growth, as generated by *Vennmaster* (23). The numbers of genes in the intersection sets are indicated. E, early exponential growth phase, M, mid-exponential growth phase, T, transition phase; S, stationary phase.

phase, 114 genes exclusively in the transition phase, and 50 genes exclusively in the stationary growth phase. Some examples of regulation over time are discussed below.

The strongest repression by CcpA was observed for the *galPMKTE* and *mtlARFD* operons, both of which are involved in the uptake of carbon sources (the monosaccharide galactose [14] and the polyol mannitol [10], respectively). Repression of both of these operons by CcpA occurred throughout the first three growth phases (early exponential phase, mid-exponential phase, and transition phase), as shown in Table 3, and part of the *mtl* operon, *mtlARD*, was repressed even in the stationary phase. Repression of the trehalose utilization operon, llmg0453, llmg0454, *trePP*, and *pgmB* also occurred in the first three growth phases (early exponential phase, mid-exponential phase, and transition phase), although the level of repression was lower than that for *galPMKTE* and *mtlARFD*. Repression of a putative cellobiose utilization system, llmg0431, llmg0432, *ptcB*, *ptcA*, llmg0439, *ptcC*, and *bglA* occurred only in the first two growth phases. The genes for utilization of glucose (*pfk*, *pyk*, *ldh*, and *pgiA*) and fructose (*fruACR*) were activated mainly in the early exponential phase.

The *arc* cluster, which was repressed by CcpA (Table 3), encodes components of the arginine deiminase (ADI) pathway and is responsible for uptake and degradation of the amino acid arginine (5, 27). Analysis of the promoter region and structural genes of the *arc* operons revealed the presence of multiple *cre* sites. The *arcABD1C1C2* cluster was repressed during the early exponential and mid-exponential growth phases, and carbon catabolite repression was relieved or overcome in the transition phase (Table 3). Analysis of the normalized gene expression levels, as measured on the DNA microarrays, suggested that the level of expression of *arc* in the Δ*ccpA* strain was lower during the transition phase than in the exponential phase of growth. Surprisingly, the arginine biosynthetic pathway, represented by the *argGH* and *argCJBDF* operons, was also more highly expressed in *L. lactis* MG1363Δ*ccpA* than in its parent strain during the exponential growth phase, although *cre* sites could not be detected in the *argCJBDF* operon.

Another example of growth phase-dependent regulation is the derepression of purine biosynthesis in *L. lactis* MG1363Δ*ccpA*. Nearly all genes in the genomic region containing the purine biosynthetic operons were upregulated exclusively during the transition phase in *L. lactis* MG1363Δ*ccpA* (Table 3). It has been suggested previously that CcpA could play a role in the regulation of purine biosynthesis on the basis of an in silico genome analysis, but so far no experimental evidence has been presented to support this claim (15). The presence of *cre* sites in the promoter regions and in some purine biosynthesis genes indicates that the observed regulation may be direct.

Functional *cre* sites are preferentially present on one side of the DNA helix relative to the promoter sequence. To determine whether the changes in gene expression observed after deletion of *ccpA* were caused by direct interactions of *L. lactis* CcpA with the affected genes or by indirect effects, the genome sequence of *L. lactis* MG1363 was searched for the presence of *cre* sites. In several cases no *cre* site was detected in the promoter regions or in the open reading frames of the affected genes, suggesting that the effects were indirect. The opposite was also observed: a putative *cre* site was present without evident regulation. It is possible that the latter genes were not expressed under the conditions used, but it is also possible that the *cre* site needs to be at a specific location in the promoter region to be functional, as has been described previously for *B. subtilis* (25). To examine this possibility, the locations of the predicted *cre* sites were determined relative to the putative TSSs, which were based on the presence of −35 hexameric sequences and/or canonical −10 sequences (Table 3). The results suggest that there is a dependence on the helix side of functional *cre* sites. The strongest repression was observed when the center of a *cre* site was located at positions −39, −26, −16, 5, and 15 relative to the TSS, all of which were separated by approximately 10.5-bp increments (i.e., a full helical turn). When the *cre* box was further upstream, repression was observed only at position −44. Repression also occurred downstream of position 15, but the putative *cre* sites were not present at 10.5-bp intervals (Table 3). Analysis of the functional *cre* sites showed that the *L. lactis cre* consensus sequence is WGWAARCGYTWWMA, which is very similar to the *cre* consensus sequence found by Miwa and coworkers (WWTGN AARCGNWWWCAWW) (35); although shorter, additional conserved sequence around the 14-bp *cre* site was not detected.

DNA helix face-dependent activation by CcpA. Activated genes containing a *cre* site upstream of the putative −35 sequence in their promoter were observed mainly in the early exponential growth phase, implying that activation by CcpA could be dependent on the growth phase.

To investigate whether the location of a *cre* site in a promoter region has an effect on activation of expression of the downstream gene, the presence of *cre* sites was determined for genes that were significantly upregulated in the exponential phase of growth in *L. lactis* MG1363 compared to the expression in *L. lactis* MG1363Δ*ccpA*. Subsequently, the locations of the putative *cre* sites were determined either on the basis of the

TABLE 3. Genes with significantly altered expression as a result of the *ccpA* mutation

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
C. Energy production and conversion									
pdhD	−3.2	−2.3			C				
pdhC				−6.7	C				
pdhB				−6.7	C				
nifJ	−2.6				C				
llmg0559			1.8		C				
glpD	−5.4	−6.4			C				
glpK	−2				C	−43	−1	AGTAACCGTTTACA	TTGACATCTTTCTGTTAT CATACTATAAT
llmg1127		−2.2			C	−273	0	AGAAATCGCTAACA	ATGCAATCTTAATAAAAA TATTGATAAAAA
llmg1726			−3.1		C				
noxB				2.1	C	−76	−38	TGTAACAGTTTACA	TTTACAAATTGCTCTAAA AAGGATATAAT
noxA			−3		C				
cydB			1.8	2.1	C				
cydA			1.8		C	207		AGTAACCGGTATTA	
llmg1915	−3				C	−61	−2	TGAAAGAGCATACA	TTGAAAATCTAAATGAAT CGGCTTATAAT
llmg1916	−8.8			2.9	C	215		TGACAGAGTTTTCA	
atpD				2.8	C				
atpG				2.2	C				
atpA				3.3	C				
ackA1			−3.4		C	218		TTAAAACGTTTGAA	
ackA2	−2				C	−22	6	ATAAAACGCATACA	ATGAAATCAACTCTAAAG TATGATATAAT
adhE	−2.5		−2.1	−2.5	C	−127	22	ATAAAGCGATTTCT	TTGCCAAATCTTTAAAAG GATTGGCAATAT
gntK	−2.3				C				
trxH				3.3	OC				
E. Amino acid transport and metabolism									
argG	−4.4	−10.1			E	−305	−37	AGTAAGCGTATTCA	TATTCAATACTATCATTT TTCTTATATAAT
argH	−3.2	−16.9		5.3	E				
llmg0184	−3.2				E				
phnC			−3.1		E				
phnB			−2.1		E				
llmg0376	1.8				E				
lysQ			2.2		E				
llmg0507			−2.6		E				
argE	−2.2	−6.8			E				
glyA			−2.3		E				
oppA				2.4	E				
pepQ	1.7		−1.5	1.3	E	−127	−66	TGTAAACCTTTTCA	GTGATTTTTCAATAAAAA TAGCGTAGAAT
trpD			3		E				
trpF			2.8		E				
trpB			2.6		E				
busAA			−2.4		E	1062		TGTAAGAGATATCT	
llmg1299			1.9		E				
telA				2.9	E				
argF		−7.4	1.9		E				
argB	2.3	−7.1	1.8		E				
argD		−7.2	1.8		E				
argJ		−8.1			E				
argC		−8.5			E				
cysK			−3		E				
metC			−4.2		E				
pheA			2		E				
llmg1993		2.3			E				
pepT				6.4	E				
oppA2				2.6	E				
arcC2	−3	−2.5		2	E				
arcC1	−2.1			1.9	E				
arcD1	−2.6	−2			E				
arcB	−1.6	−1.5	−1.1		E	−155		TGAAAACTTTTACA	
arcA	−3.8	−2.9			E	−43	4	TGTAAACGATTCCA	TTGACAAAAAATATGCAT AGATGTATAAT
llmg2477		3.5			E				
glnA			−2.2		E				

Continued on following page

TABLE 3—*Continued*

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
carB	2.6				EF				
trpE			3.9		EH	195		TGAAATCATTAGCA	
oppD			−2.2		EP				
oppB2				1.9	EP				
llmg1195		−3.5			ER				
butB	−3.7	−5			ER				
gltS	−2.6	−7			ET				
gntP	−4.5		1.9		GE	−54	71	TGTAAGCGCAAGCA	TTCAAAGAAAATTTGAAA AAGACTAAATT
pmrB		−14.5			GEPR				
llmg0856	1.8				GEPR				
llmg2513		−2.5			GEPR				
F. Nucleotide transport and metabolism									
nrdD	1.9		−2.4		F				
udk	2.1				F	−85	−66	TGTGAACGCTTAAA	ATGACAAAAAGCCCAAAA ATTGTGGTAGAAT
pyrR	1.9				F				
pyrP	2.4				F				
pyrB	1.9				F				
purC			−5		F				
purQ			−3.2		F	262		TGTAATGGATTTCA	
purI			−3.4		F				
purM			−4.5		F				
purN			−5.9		F				
hprT			−3.1		F				
purH			−10.2		F				
purD			−6.3		F				
purK			−2.1		F				
pyrDB	2.1				F				
guaC			−4.9		F				
pyrC	2.1				F				
pyrE	2.4				F				
nrdE				2.4	F				
gmk			−3.4		F	84		TGAAAGTGATAACA	
purA			−3.1		F	309		TGCACACGTTATCT	
adk			−2		F				
purR		2	−2		F				
carB	2.6				EF				
G. Carbohydrate transport and metabolism									
mtlA	−4.9	−10.3	−8.5	−2.5	G	−44	−17	TGGTAGCGGTTATA	TTTACAGTCTTATTGGTA GCGGTTATAAT
mtlF	−5.3	−7.6	−5.9		G				
mtlD	−17.2	−20.9	−15	−2.2	G	−81	40	TGTAAAAGCTTACA	TTGAAAATTAATCAATAA AATCAAAAAAAT
ptsH			−2.2		G				
glgB	−3.3	−2.6			G	−58	−28	TGAAAACCTTTGCA	TATGAAAACCTTTGCAAA AGTGCTAAAAT
ptcB	−5.6	−4.4			G	−62	−16	TGAAAACGTTATAA	TTGCATTATGAAAATGAA AACGTTATAAT
ptcA	−5	−3.3			G				
ptcC	−4.3	−2			G	−96	−40	AGAAACCGCTTTCT	TTCTTTACTTTGCCTATT AATGCTATAAT
bglA	−3.1				G				
femD				2.5	G				
llmg0453	−4.2		−2.5		G	−64	−26	TGAAAACGTTTTTA	TTGCTGAAAACGTTTTTA TGTGATACAAT
llmg0454	−5.7	−3.7	−2.4		G				
trePP	−13.5	−2.8			G				
llmg0487	−3.2				G				
llmg0488	−2.8				G				
llmg0489	−2.1				G				
llmg0490	−2.3				G				
malG	−2.9	−2.3		2.8	G				
malF	−3.4	−2.4			G				
malE	−2.8	−2.5		2.6	G	−24	13	ATAAACCGTTTTCA	TTGACAAAAAGCAAACGG TTGCGTAAAAT
dexA				2.5	G				
amyY				1.8	G				
agl				4.1	G				

Continued on following page

TABLE 3—*Continued*

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
mapA				4	G				
ascB	−9	−4.4			G	−64	−39	TGAAATCGCTATCA	TATCAAAAACAATTAAAA AATGGTAAAAT
llmg0870	1.9				G				
rpe2			5.5		G	−168	−104	AGTAATCGTTATCT	TTGACAGGTTGTTTTCCT GATGATATAAT
rpiB			7.9		G				
llmg0959			8.3	2.1	G	462		TGAAACAGTTATCA	
llmg0960			5.8		G				
glpF2	−5.1	−3.9			G				
pfk	1.9				G	−134	−56	TGAAAACGTTT-CA	AAGAGATTTTTTTATAAA TACGTGATATAAT
pyk	2.1				G				
ldh	2.4				G				
llmg1468	−3.5	−2.6			G	−24	2	ATAAAGCGTTTACA	TAAAAAGTAAAGGCCAAT TATGTTATAAT
fruA	2.9		3.3		G				
fruC	3		3.9		G	−345		TGAAAAAGGTAACA	
pmi			−3.6		G	−29	12	TGAAAGCGAATAAA	TTGTTCTAGATAAATTTT GTGATATAAT
gpmC			1.8		G				
chiC	−8.1	−4.5	−2.3		G	−55	1	AGTTAGCGCTTACA	TTTAAGTTAGGGAAAAAA ATTGTTAGAAT
galT	−11.3	−8.3	−7		G				
galK	−16.9	−10.7	−6.2		G	38		TATCAGCGTTAACA	
galM	−15.2	−10.4	−5.5		G				
galP	−18.9	−10.3	−5.1		G	360	3	TGCCATCGTATTCA	TTGGAAACCCTTTCTTAA ACCAAAGTGTTATACT
bcrA				1.9	G				
glpF3	2				G				
llmg2431	−2.1				G	−56	−27	TGAAAACGCTTTTT	CTGAAAACGCTTTTTTTG TGATAAAAT
pgiA	1.9	1.9		2.1	G	−139	−56	AATAAGCGCTTACA	TTGTTAAAAAGCCGAAAA AGTGATAAAAT
glcU	2.8				G				
gntP	−4.5		1.9		GE				
pmrB		−14.5			GEPR				
llmg0856	1.8				GEPR				
llmg2513		−2.5			GEPR				
llmg0961			8.6		KG				
fruR	2.5				KG	440		TGAAAAAGGTAACA	
H. Coenzyme metabolism									
lplL	−2.2		−2.8		H				
menA	1.8				H				
thiI		2.5			H				
gltD	−2			2.5	H				
dltD			1.9		H	−375		TGTAAGTATTTTCA	
ribH		−4.8	1.9	2.2	H				
ribA		−5.2			H				
rib			1.9		H				
ribD		−2.7			H				
pnuC2	2.9				H				
folD			−3.1		H				
kdtB			−2.1		H				
trpE			3.9		EH				
I. Lipid metabolism									
mvk			−2.9		I				
llmg0431	−2.3	−2.2			I				
llmg1517			1.9		I				
accA			2.3		I				
accC			2.8		I				
butA	−4.4	−4.7			IQR				
llmg1762			−2.8		IQR				
J. Translation, ribosomal structure, and biogenesis									
llmg0015		2.1			J				
rpmG			−2.3		J				
prmA			2		J				
tgt		2.9			J				

Continued on following page

TABLE 3—*Continued*

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
rpsD				2.1	J				
tyrS				5.9	J				
thiM		−2.2			J				
hisF			1.9		J				
truB			−2.5		J				
rplU				2.3	J				
rpsA			−3.3		J	−169		AGAAAATGTTTTCA	
trmU	2				J	906		TGCAAGCGATTTGA	
pnpA			−2.2		J				
thrS	2.4				J				
argS			−2.6		J				
rplO			−2.2		J				
proS				2.3	J				
valS				2.1	J				
K. Transscription									
mtlR	−4.2	−11.5	−6.7	−2.4	K				
llmg0141		−3.4			K				
cspE			−3.4		K				
llmg0432	−2.5				K				
llmg0439	−10.8	−4.7			K				
lytR			−2.6		K				
malR				3.4	K				
ccpA	4.5	3.7	5		K	−65	−27	TGATATCGCTTCCA	TTGAAAAGGTTTACAGTT CATGATATAAT
llmg0956			2.5		K	−142	−108	GGTAATCGTTTACT	TTGCTAAACTTGTTTATA TATGAGAGAAT
rplL				2.3	K				
cspD			−5		K				
gidC			−2.2		K				
llmg2218		2.6			K	−52		TGAAAGCGCAAACA	
glnR			−2.1		K				
llmg0961			8.6		KG				
fruR	2.5				KG				
llrB			−2.5		TK	−215		AAAAAGCGTATTCA	
L. DNA replication, recombination, and repair									
llmg0151				3.3	L				
matR	−2.9				L				
ltrC			2		L				
tnp-981			2.1		L				
xseB			−2.3		L				
xseA			−2.2		L				
llmg2007			1.9		L				
llmg2444			−2.1		L				
rnhA			−2		L				
M. Cell envelope biogenesis, outer membrane									
rgpA	−2.1				M				
pbp2B		1.9			M				
mgtA		1.9			M				
galE	−2.4	−2.4	−2.3		M				
llmg2320				−3.4	M	1694		TTAATACGATTACA	
O. Posttranslational modification and protein turnover									
gcp				1.9	O				
ahpF				1.9	O				
groEL2				−2.7	O				
tig			−4.1		O				
clpB			2.4		O				
llmg1129		−2.1			O				
dnaK				−2.5	O				
fabD		1.8			O				
pepO2			2		O				
trxH				3.3	OC				
clpP				4.1	OU	−169	−101	TGAAAGCGTTAAGA	TTGACCTTTTTTGACCAA TGAGTTATAAT

Continued on following page

TABLE 3—*Continued*

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
P. Inorganic ion transport and metabolism									
phnD			−6.5		P	−269	−27	TGTAAAGGCTTAAA	TTGTAAAGGCTTAAAAAA AGGGTTAGAAT
phnE			−2.6		P				
plpA	2.2				P				
plpB	1.9				P			<	
fhuD	2.2				P	821		TTAAAGCGGTTAAA	
llmg0514			−2.1		P				
llmg0661			1.9		P	776		TGAAAACTCTAACA	
fur			1.9		P				
cspD2			−3.5		P				
orf53				2.5	P				
fdhC	−2.6				P	−20	14	TAAATACGCTTTCA	TAGCATTCTAATATAATT TATGATAGAAT
pstC				2.5	P				
oppD			-2.2		EP				
oppB2				1.9	EP				
pmrB		−14.5			GEPR				
llmg0856	1.8				GEPR				
llmg2513		−2.5			GEPR				
Q. Secondary metabolite transport and metabolism									
llmg1629			−2.1		QR				
butA	−4.4	−4.7			IQR				
llmg1762			−2.8		IQR				
R. General function prediction only									
llmg0146	−2.5				R				
llmg0167				2.5	R				
llmg0185	−4.3	−2.8			R	8	6	TGAATACGAATACA	TTCTTATTTCTACAGTTT TGTGTTAAAAT
llmg0194		1.8			R	−38	84	TGAAATCGTTCACA	TTGTCAAAGATTAAAAAT TATCGTAGAAT
pgmB	−14	−3.6	−2.1		R				
llmg0481				2.5	R				
llmg0541			−2.2		R	−30	2	TGTAAGCGTTGATA	TGTTTTTTTTTATTAAAAA AATGTTATAAT
llmg0736				−3.7	R				
llmg0761			−2.3		R	−210		TTTAAGCGTTCACA	
llmg0765			−2.9		R				
llmg0876				2.4	R				
llmg0995			−2.1		R	24		TGATATCGATAACA	
bmpA			−2		R				
orf18			1.9		R				
cpo			−2.6		R				
lrgA	2				R				
llmg1912			−2.4		R				
pbuO		1.9	−3.5		R				
llmg1920			−2		R	487		TTTAAACGTTTACC	
adhA	−3.1				R	−36	8	AGAATGCGTTTACA	GATACAAAACGAGGTGAA AAGTGTTATAAT
llmg2436	−2.4				R				
llmg1195		−3.5			ER				
butB	−3.7	−5			ER				
pmrB		−14.5			GEPR				
llmg0856	1.8				GEPR				
llmg2513		−2.5			GEPR				
butA	−4.4	−4.7			IQR				
llmg1629			−2.1		QR				
llmg1762			−2.8		IQR				
S. Function unknown									
llmg03303		−2.7	−2		S				
llmg0334		−2.9			S				
llmg0343			2		S				
llmg0448				2.4	S				
llmg0476	2.1				S				
llmg0492			−2.6		S				
llmg1202	−2.2				S				
llmg1540	2.1				S				

Continued on following page

TABLE 3—*Continued*

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
llmg1666			2.1	1.8	S				
llmg1917	−4.2				S				
llmg1936			1.9		S	428		TGTCAGCATTTTCA	
llmg2164				−2.7	S				
llmg2194			−2.3		S				
chb	−10.3	−4.4			S				
llmg2213			1.8		S				
llmg2337				2.2	S				
llmg2338				2.6	S				
T. Signal transduction mechanisms									
kinC				3.7	T				
cstA	−11	−7.1	−2.8		T	−66	5	TGTAATCGGTTACA	TTATCTTTTTGATTAAAT AGTGCTAAAAT
kinF				2.3	T				
kinE			1.8		T				
uspA	−2.3	−2			T				
llmg2047			1.9		T				
llrB			−2.5		TK				
gltS	−2.6	−7			ET				
U. Intracellular trafficking and secretion									
llmg1391			2.2		U				
cluA		2			UW				
ps457			2.1		UW				
clpP				4.1	OU				
V. Defense mechanisms									
hsdS			1.9		V				
llmg0989	−4.8	−2.3			V	1488	1470	TGAAAGCACTGCCA	TATTTACTTTTGGAATTT AAATGCTACAAT
hdiR			2.1		V				
llmg1467	−2.7	−2.2			V				
lmrA			1.9		V				
W. Extracellular structures									
cluA		2			UW				
ps457			2.1		UW				
X. No prediction									
llmg0183	−2.5		−2.1		X	−67	−39	TGAAAGTGCTTGCA	TTGCAAAGAATCTTTAAT CTTGCTAGAAT
llmg0311			−2.8		X	−29	−31	TGATAACGCTGACA	TTGATAACGCTGACAAAT TTTCTGCTATAAT
llmg0379			−2.1		X				
llmg1090	−4.7	−2.8			X	−94	−44	AGAAAGCGTTTTAT	TAGACTTAAGTTAGAAAA TAGGTTATAAA
llmg1091	−4.5	−3.5			X				
llmg1092	−4.2	−2.6			X	791		TGCGAACGCATTCA	
llmg1093	−3	−2.4			X				
llmg1094	−22	−4.5		2.7	X				
llmg1095	−5.3	−3.1			X				
llmg1096	−8.8	−3.5		2.4	X	470	484	AGAAAGCGGACTCA	TCGCAAGAGAGGCTCAAA TTTTATTAAAAT
llmg1108			2.4		X				
panE		1.8			X				
potD			2.4		X				
orf46			2.7		X				
orf30			2.2		X				
orf28			1.8		X				
orf26			1.9		X				
orf22			1.9		X				
orf20			2		X	530		TAGAAGCGATAACA	
llmg1563				−2.2	X				
llmg1750				2.3	X	313		GGCAAACGTTATCA	
llmg1773				1.9	X				
llmg1944				1.8	X				
llmg2036			3.6	2.5	X				
ps431				2.3	X				
ps412	−2				X	19	315	TGTATGCGTTTTAA	ATTTCAAATTTTTAGGCA TTCGTGGTATAAT

Continued on following page

TABLE 3—*Continued*

Gene	Upregulation or downregulation[a]				COG	Postion 1[b]	Position 2[c]	*cre* sequence	Putative −35 and −10 sequence
	E	M	T	S					
llmg2144	−2.5	−2.3			X				
llmg2145	−2.3			2.3	X				
llmg2146	−2.3	−2			X				
llmg2211	−2				X	−9	33	AGAAAGGGTTTTCT	TGTGTTATAAT
llmg2286	−3.6	−2.4			X	−31	−16	AGAAAGCGCTATAA	TTGTCATCTATAAAAGAA AGCGCTATAAT
llmg2482			−2.3		X				
llmgpseudo54	−3.1	−3.2			X				
llmgpseudo69	−2.5		−2.1		X				

[a] Positive values indicate upregulation, and negative values indicate downregulation. E, early exponential phase; M, mid-exponential phase; T, transition phase; S, stationary phase.
[b] Position relative to the (putative) TSS.
[c] Position relative to the start of the gene.

position of putative −10 and −35 regions or on the basis of the position of transcription start sites determined experimentally. The centers of the putative *cre* sites were located at position −56 or −66 in the promoter regions of genes which were probably activated by CcpA in the early exponential phase of growth.

Autoregulation of *ccpA* expression. We detected a *cre* site that encompasses the −35 sequence in the promoter of *L. lactis ccpA*, suggesting that the regulator may be subject to autoregulation. Although previous results obtained using a *ccpA* disruption strain indicated that CcpA does not regulate its own gene (32), a recent report suggested that the disruption mutant used by Luesink and coworkers does not fit the proper Δ*ccpA* phenotype (12). This would also explain the partial derepression of the *gal* operon (32), which is not in accordance with the finding that there was complete derepression of *gal* in *L. lactis* MG1363Δ*ccpA* (Table 3). To examine whether *L. lactis* CcpA regulates itself, the promoter of *ccpA* (P*ccpA*) was cloned upstream of the promoterless *E. coli* β-galactosidase gene in pILORI4, and enzyme assays were performed (Fig. 3C). The 2.5-fold-lower β-galactosidase activity observed in *L. lactis* MG136 than in *L. lactis* MG1363Δ*ccpA* showed that CcpA is subject to autoregulation in *L. lactis*. To rule out any indirect effects, EMSAs were performed using P*ccpA* as the probe (Fig. 4A). A more slowly migrating band of the probe in the presence of nanomolar concentrations of purified H6-CcpA protein indicated that there was a direct interaction between H6-CcpA and P*ccpA*. As a negative control, EMSAs were performed with the promoter region of a gene not regulated by CcpA, as determined by the DNA microarrays. The probe (Pllmg1650) did not exhibit any binding of H6-CcpA (Fig. 4B).

***pepQ* is activated by CcpA.** *pepQ* is one of the genes that was downregulated in *L. lactis* MG1363Δ*ccpA*. In nearly all LAB the *pepQ* and *ccpA* genes are in a tail-to-tail orientation (Fig. 3A; see Fig. 1 posted at http://molgen.biol.rug.nl/publication/ccpA_data). The *pepQ* and *ccpA* promoters share a *cre* site, which might have a function in both activation of *pepQ* and repression of *ccpA*. Such a phenomenon has been described for *Lactobacillus delbrueckii* and *Lactobacillus pentosus* (34, 44).

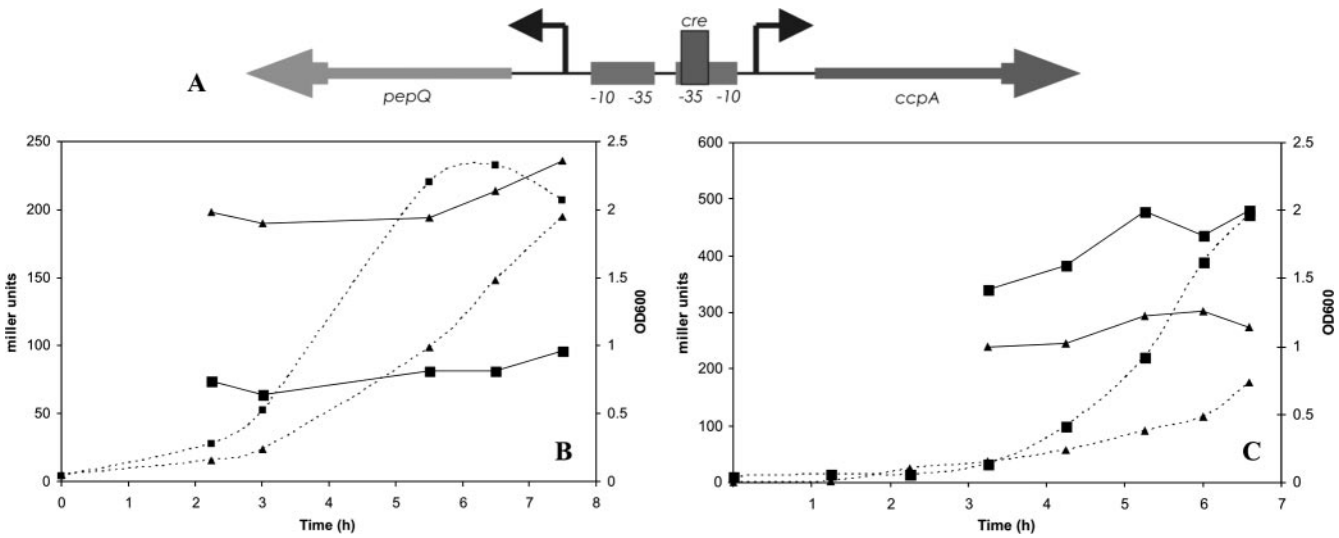

FIG. 3. (A) Genetic structure of the *pepQ-ccpA* region. (B) Growth of (dashed lines) and β-galactosidase activities in (solid lines) *L. lactis* MG1363 (■) and *L. lactis* MG1363Δ*ccpA* (▲), both containing pILORIQZ. (C) Growth of (dashed lines) and β-galactosidase activities in (solid lines) *L. lactis* MG1363 (■) and *L. lactis* MG1363Δ*ccpA* (▲), both containing pILORIAZ. All four strains were grown in GM17 at 30°C. OD600, optical density at 600 nm.

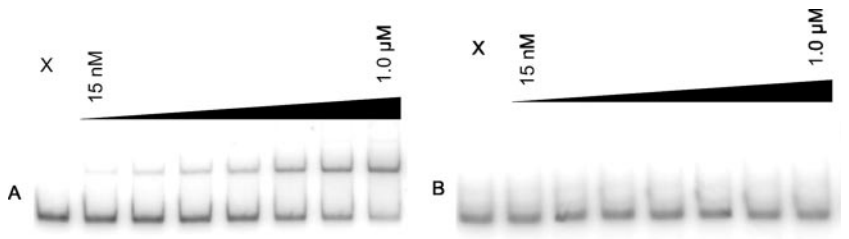

FIG. 4. Electrophoretic mobility shift assays of H6-CcpA interactions with P*ccpA* (A) and Pllmg1650 (B) (negative control). DNA fragments from both promoter regions were obtained by PCR (see Materials and Methods) and end labeled with ^{32}P. Lane X contained the probe without added protein. The remaining lanes contained probe samples incubated with increasing concentrations of H6-CcpA (concentrations ranging from 15 nM to 1 μM). For each successive lane from left to right the concentration of H6-CcpA was doubled.

However, activation of *pepQ* by carbon catabolite control has not been observed in *L. lactis* (16). *L. lactis pepQ* is preceded by a putative ribosome binding site (GGAGG) with a $\Delta G°$ value of −14.4 kcal/mol (60.2 kJ/mol). Upstream of the ribosome binding site, there is a promoter-like structure consisting of a −35 hexanucleotide (GTGATT), a 17-bp space, and a −10 sequence (TAGAAT). Primer extension analysis showed that transcription starts at the adenine residue 5 bp downstream of the −10 hexanucleotide (data not shown). The putative *cre* site is located upstream of the −35 sequence of *pepQ* at position −66 relative to the *pepQ* transcriptional start and encompasses the −35 sequence of P*ccpA* (see above). Northern blot analysis of 16S rRNA and *pepQ* mRNA showed that transcription from the *pepQ* promoter was significantly reduced in *L. lactis* MG1363Δ*ccpA* (Fig. 5).

PepQ activity assays (not shown) and β-galactosidase activity assays (Fig. 3B) showed that both the activity of PepQ and the *pepQ* promoter activity were twofold lower in *L. lactis* MG1363Δ*ccpA* than in *L. lactis* MG1363. These observations complement and confirm the DNA microarray results.

Binding of purified H6-CcpA to the *ccpA*/*pepQ* promoter region (Fig. 4A) indicated that CcpA might have been responsible for the positive effect on *pepQ* transcription and that this might have been caused by direct activation of the promoter of *pepQ*.

DISCUSSION

The research described in this paper demonstrated the importance of CcpA as a global regulator in *L. lactis*, as deletion of CcpA from the genome of *L. lactis* changed the expression of nearly 13% of all the genes in the genome of *L. lactis* during growth. As we discuss below, CcpA appears to be involved not only in regulation of carbon metabolism but also in regulation of glycolysis, nucleotide metabolism, and nitrogen source uptake and degradation and may have an effect on the intracellular pH because of regulation of the ADI pathway.

Deletion of the *ccpA* gene resulted in a lower rate of growth of the *L. lactis* mutant and upregulation of a large number of genes, corroborating the hypothesis that CcpA has a role as a pleiotropic repressor. For a number of genes the level of expression was lower in *L. lactis* MG1363Δ*ccpA* than in its parent, revealing that expression of these genes is activated by CcpA. The greatest direct effects were observed in and around the exponential phase of growth. Activated genes containing a *cre* site upstream of the putative −35 sequence in their promoter were observed mainly in the early exponential growth phase, while most repression by CcpA took place in the transition phase between the exponential and stationary phases of growth. The effects of the *ccpA* mutation on gene expression in the stationary phase were less pronounced. The majority of the

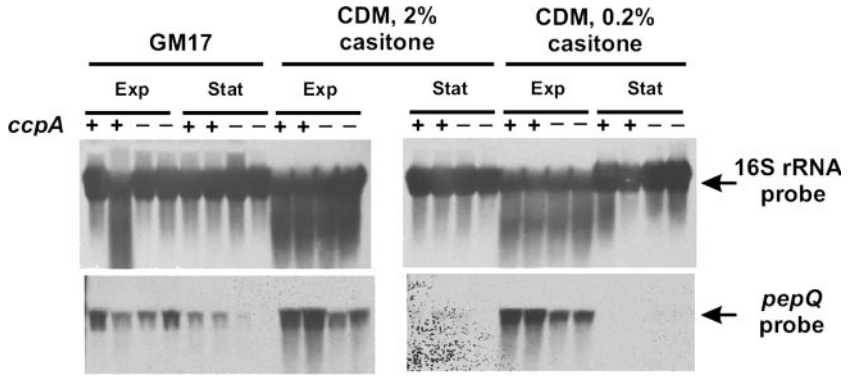

FIG. 5. Northern blot analysis of *pepQ* transcription in *L. lactis*. A 16S rRNA-specific probe was used to examine the quantity of RNA in samples of *L. lactis* MG1363Δ*ccpA* (CcpA$^-$) and *L. lactis* MG1363 (CcpA$^+$). The strains were grown in different media. GM17 and CDM containing 2% Casitone are peptide-rich media, and CDM containing 0.2% Casitone is a nitrogen-poor medium. Exp, exponential phase of growth; Stat, stationary phase. Duplicate samples were used in all analyses.

effects observed in the stationary phase may have been indirect because *cre* sites were detected infrequently in the promoter regions of the affected genes in the stationary phase. The change in expression may have been caused by the growth of the bacterium without CcpA-mediated carbon catabolite control, which has an effect on the composition of a cell and also on the composition of the medium. After several hours of growth of *L. lactis* MG1363Δ*ccpA* the cells likely had taken up and metabolized different compounds (for instance, glucose and arginine) compared to the parent strain, altering the medium composition, which had an effect on the growth of the organism.

An example of differential expression in *L. lactis* MG1363 and its isogenic *ccpA* mutant during growth is the expression of the ADI pathway, which was derepressed in *L. lactis* MG1363Δ*ccpA* during the exponential phase of growth, whereas it is typically more active in the stationary phase in *L. lactis* MG1363. Normally, ADI is active only when glucose is depleted and arginine is present (27). Perhaps medium arginine was depleted earlier in the Δ*ccpA* strain through derepression of ADI, which, as observed, would decrease the expression of the *arc* operon in the stationary growth phase, since the activity of the ADI pathway is dependent on the concentration of arginine (27). This hypothesis would explain the overexpression in *L. lactis* MG1363Δ*ccpA* of the *argCJBDF* operon in the exponential phase of growth, which is derepressed under low-arginine conditions (17, 27). These two operons, which are required for the biosynthesis of arginine via glutamate, were upregulated during exponential growth of *L. lactis* MG1363Δ*ccpA*, although in both operons no *cre* sites could be detected. The ADI pathway protects the cell against acid stress by increasing the internal pH, and it is possible that the different expression of the ADI pathway in *L. lactis* MG1363Δ*ccpA* had an effect on the internal pH. When arginine is depleted early in growth, which might be the case in *L. lactis* MG1363Δ*ccpA*, a cell is less protected against acid stress because it is unable to utilize the ADI pathway.

Recently, it has been found that *B. subtilis* CcpA interacts with RNA polymerase to inhibit transcription (25). Such an interaction might also take place in *L. lactis* because of the conserved locations of functional *cre* sites at a specific side of the helix. When the *cre* box is further upstream of the −35 sequence, repression is observed only at position −44. Repression also occurs downstream of position 15, but the putative *cre* sites at the locations are not present at 10.5-bp intervals. Regulation by a roadblock mechanism has been suggested to take place in *B. subtilis*, in which the presence of CcpA on the DNA blocks transcription (6, 19). Since the requirement for *cre* localization on a specific side of the DNA helix for a functional *cre* site is eliminated when *cre* is further downstream than position 15 relative to the TSS, there may also be a mechanism for a roadblock in CcpA regulation in *L. lactis*.

B. subtilis CcpA activates transcription of the *ackA* gene when the *cre* site is present at positions −56.5 and −66 (49), suggesting that activation of transcription by CcpA is helix face dependent, probably because interaction with the alpha subunit of RNAP is necessary for the activating properties of CcpA. It is also possible that CcpA bends the DNA, allowing the α-subunit of RNAP to interact with UP sequences, very rich in AT (8), to enhance transcription. A similar mechanism for transcription activation has been suggested for the *E. coli* homolog of CcpA, Cra (or FruR) (38, 41). Mutations in the AT-rich region upstream of the −35 sequence in the promoter of *B. subtilis ackA* eliminated activation by CcpA (36). Highly AT-rich sequences are also present upstream of the (putative) −35 and *cre* sites of all four activated genes in *L. lactis* (Table 3), indicating their importance.

There is a conserved *cre* site encompassing the −35 sequence in the *L. lactis ccpA* promoter. It has been suggested that autoregulation of *ccpA* takes place in several other LAB (34). β-Galactosidase activity assays of a strain of *L. lactis* carrying a fusion of *E. coli lacZ* to the *ccpA* promoter showed that the level of activity of P*ccpA* is higher in *L. lactis* MG1363Δ*ccpA* than in *L. lactis* MG1363. Furthermore, EMSAs revealed that there is a direct interaction between H6-CcpA and a DNA fragment carrying P*ccpA*, suggesting that *L. lactis* CcpA regulates its own expression. Autoregulation of CcpA might be a way for the cell to carefully balance the amount of CcpA in the cell.

The *cre* site encompassing the −35 hexamer of P*ccpA* might have a second function in activation of the divergently transcribed *pepQ* gene. The highly conserved divergent orientation of *pepQ* and *ccpA* in all LAB suggests that there is a link between the regulation of *pepQ* and the regulation of *ccpA*. Indeed, such a relationship between *pepQ* and *ccpA* has been described for *L. delbrueckii* and *L. pentosus* (34, 44). The twofold-lower PepQ activity in *L. lactis* MG1363Δ*ccpA* than in *L. lactis* MG1363 and the twofold-lower strength of its promoter, measured using β-galactosidase reporter activity assays, suggest that there is also a *ccpA-pepQ* link in *L. lactis*. Binding of H6-CcpA with the DNA fragment carrying the partially overlapping *pepQ* and *ccpA* promoters proves that there is a direct interaction. Thus, we concluded that CcpA has a positive effect on the transcription of *pepQ*, which might be caused by direct activation of P*pepQ*. One of the substrates of *L. lactis* PepQ, the dipeptide leucylproline, has an effect on the expression of *prtP*, *pepC*, *pepN*, and the *opp-pepO1* operon (16). Recently, it has been found that leucine, one of the products of the hydrolysis of leucylproline by PepQ, enhances binding of the pleiotropic nitrogen regulator CodY to its operator site (7), suggesting that PepQ has a role in regulation of the proteolytic system of *L. lactis*. The data presented here and the presence of such a conserved genetic organization in many other LAB may indicate that there is a general method used by this group of microorganisms to couple carbon metabolism to nitrogen metabolism. Regulation of *pepQ* by CcpA in LAB has been suggested by Mahr et al. (34) and has been shown to operate in *L. delbrueckii* subsp. *lactis* by Schick et al. (44), but only now with the application of DNA microarray technology can we begin to understand the possible significance *pepQ* regulation by CcpA and the resulting intertwinement of carbon metabolism and nitrogen metabolism.

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